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The goal of this grant was to develop a prototype epidermal biosensor for carcinoembryonic antigen (CEA). An epidermal biosensor was conceived as a new approach for the early continuous, in vivo detection of the onset of disease by the using genetically modified skin cells to respond to molecules secreted by tumor cells.

The research we have conducted has allowed us to conclude that human keratinocytes in vitro can be engineered to express a chimeric cell surface receptor and that these modified cells could recognize and bind CEA. Technical problems in generating chimeric receptor constructs were resolved but we were unable to create a chimeric cell surface receptors that had domains that would bind and respond to CEA in human keratinocytes.

In the absence of supplemental funding, we were unable to pursue other avenues for constructing cell surface receptors that would introduce into keratinocytes the capacity to act as in vivo biosensors. We remain confident that such chimeric receptors can be generated to respond to tumor antigens such as CEA. We believe that keratinocyte biosensors could be effective early detection systems and play an important role in the management of breast cancer.

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INTRODUCTION

The goal of this grant was to determine if it would be possible to develop an epidermal biosensor for carcinoembryonic antigen (CEA) as an early, sensitive detector of the onset of breast cancer. An epidermal biosensor represented a new concept for detection of disease whereby a small area of skin would contain keratinocytes that were genetically modified to recognize and respond to molecules secreted into the circulation by a tumor. The main goal of this proposal was to generate an *in vitro* prototype by introducing into human epidermal keratinocytes a chimeric cell surface receptor that would bind and respond to CEA. The long-term objective was to explore the use of epidermal biosensors as a continuous, *in vivo* monitors for the presence of tumor antigens expressed by breast cancer. The expectation was that epidermal biosensors would provide early detection of the onset of disease for high-risk patients so that appropriate medical management could be initiated when it is most likely to result in a positive outcome.

BODY

Statement of Work and rationale for modified chimeric receptor

The overall goal and specific aims focused on the development of an epidermal biosensor for carcinoembryonic antigen (CEA) for the early detection of breast cancer. As noted in the annual reports, we modified a control chimeric receptor that when introduced into keratinocytes bound CEA. We modified this receptor with different transmembrane (TM) and intracellular domains from the epidermal growth factor receptor (EGFR) and the tumor necrosis factor α receptor, type I, (TNF α R1). These chimeric receptors used as the ligand binding domain the single chain variable fragment (scFv) antibody to CEA.

The Statement of Work describes the tasks to develop and investigate the modified chimeric receptor for CEA.

Statement of Work and Progress - Final

Specific Aim 1. Engineer CEA-binding chimeric receptors

Progress

Technical Objectives 1. Prepare plasmid constructs

Task Generate constructs - see Table 1.

DONE

Technical Objective 2. Prepare retroviral expression vectors

Task . Generate retroviral vectors for constructs in Table 1.

DONE

Specific Aim 2. Introduce and investigate chimeric CEA-binding receptors in human keratinocytes *in vitro*.

Technical Objective 1. Transduce keratinocytes with and determine chimeric receptor expression

Task Transfect keratinocytes with retroviral vector for constructs and select transfected cells resistant to G418. **DONE**

Technical Objective 2. Measure binding of CEA to transduced cells

Task 1. Prepare stock of I-125 CEA **DONE**

Task 2. Measure binding of I-125 CEA to keratinocytes expressing construct **DONE**

Technical Objective 3. Evaluate activity in response to CEA

Task Measure response of modified keratinocytes treated with CEA. **NOT DONE**

Specific Aim 3. Examine activity of CEA-chimeric receptors in human keratinocytes *in vivo*. **NOT DONE**

Rationale for the scFv anti-CEA TNF α RI chimeric receptor in keratinocytes

The objective of this grant was to generate an effective chimeric receptor, i.e. one that would recognize and respond to CEA, and test its capacity to function in human keratinocytes to produce a cellular and tissue response in the presence of CEA. Cell surface receptors have three critical domains: an extracellular ligand binding domain, a transmembrane domain and an intracellular effector domain. Recent literature supports the concept of single chain variable fragment (scFv) antibody as a ligand binding domain in chimeric cell surface receptors [1-5]. A chimeric receptor possessing a scFv antibody that recognizes CEA has been described and a plasmid containing the construct was obtained from P. Darcy [1].

For the transmembrane and intracellular domains, we utilized the EGFR and the TNF α receptor I sequences. Keratinocytes are known to express both of these receptors. Keratinocytes express a 55kd TNF α RI that binds TNF α [6]. Keratinocytes respond to TNF α by up-regulation of IL-1 and IL-6 and other cytokines resulting in inflammation [6-8]. TNF α RI (CD120a) activation requires participation by one or more TNF receptor association factors (TRAF's) which may bring about receptor aggregation and signaling through the NF-kappaB/c-jun pathway [9,10]. Ligand binding is thought to dissociate a factor (SODD – silencer of death domains) from TNF α RI allowing other factors to associate and produce an active signaling complex. It was hypothesized that CEA binding through an scFv ligand binding domain will activate TNF α RI and trigger its signal transduction pathway.

Human epidermal keratinocytes express EGFR and respond to EGF [11]. The cDNA sequence for the EGFR has been reported and its transmembrane and intracellular domains identified [12]. It was hypothesized that CEA binding through an scFv ligand binding domain will activate EGFR and trigger its signal transduction pathway.

Tasks accomplished

1. Generation of retroviral vector containing the scFv anti-CEA Fc γ cDNA.

The pRSV anti-CEA Fc γ was obtained from P. Darcy [1]. The plasmid was grown and characterized. The plasmid was used for generating a retroviral vector for stable transduction of human keratinocytes [13]. Briefly, the plasmid was transfected into PE501 ecotropic packaging cell line and the resulting virus particles used to infect the PA317 amphotropic cell line. The PE501 and PA317 cell line were obtained from Organogenesis Inc, Canton MA. The medium from the infected PA317 cells containing the infectious, replication-defective retrovirus expressing the cDNA for scFv anti-CEA Fc γ chimeric receptor and the cDNA for neo to confer resistance to G418.

2. Transduction of human keratinocytes in vitro.

Primary human keratinocytes and HaCaT cells were transduced with the retroviral vector expressing the cDNA for the chimeric receptor. Cells expressing the chimeric receptor were selected by growth in medium containing G418.

3. Demonstration of expression of the chimeric receptor.

The cDNA sequence expressed by the retrovirus contained the c-myc tag in the intracellular domain of the chimeric receptor. Cells were stained for the presence of c-myc epitope. Figure 1 shows that the majority of transduced keratinocytes displayed bright fluorescent staining for the c-myc epitope. Untransduced cells showed no staining.

4. Preparation of I-125 CEA.

CEA was purchased from Sigma (St Louis, MO) and labeled with I-125 using the IODO-GEN tubes (Pierce).

5. Demonstration of binding of CEA to CEA-Fc γ keratinocytes.

Keratinocytes were exposed to I-125 CEA. Untransduced keratinocytes retained little radioactivity whereas transduced keratinocytes showed significant binding to I-125 CEA (Figure 2).

6. Construction of plasmids expressing scFv anti-CEA chimeric receptors

Table 1 describes the constructs and designation. The scFv anti-CEA sequence was excised from plasmid expressing construct #1 (scFv-CEA-Fc γ) and this segment extended by PCR with primers to allow insertion of the sequence into the Bgl II and Hind III sites of the retroviral expression plasmid pLNCX-2 (Clontech, Palo Alto, CA).

Table 1. Chimeric Receptors Constructs in pLNCX-2

Name	Extracellular Domain	Transmembrane Domain	Intracellular Domain
#1	antiCEAscFv	Fc- γ	Fc- γ
#2C	antiCEAscFv	TNF α RI	TNF α RI
#2D	antiCEAscFv	EGFR	EGFR
#3B	antiCEAscFv	Fc- γ	TNF α RI
#3C	antiCEAscFv	Fc- γ	EGFR

- Construction of plasmid #2C

The cDNA expressing TNF α RI has been cloned [14] and a plasmid containing the cDNA was obtained from J. Pober (Yale Univ.). The plasmid was grown and characterized. Since the sequences incorporating the transmembrane and intracellular domains had a Hind III site, the Quick Change mutation kit (Stratagene) was used to generate conservative single-base changes to nullify the Hind III sites. A primer was synthesized to allow the TNF α RI domains of the mutant to be inserted in phase through the Hind III – Sal I site of the scFv-CEA /pLNCX-2 vector. The resulting construct was transformed into DH5 α and plasmid isolated from a large scale preparation.

-Construction of plasmid #2D

The cDNA expressing ERFR has been cloned [12] and a plasmid containing the cDNA was obtained from D. Stern (Yale Univ.). The plasmid was grown and characterized. Primers were synthesized to allow the insertion of intracellular portion of EGFR into the Hind III – Sal I site of the scFv-CEA /pLNCX-2 vector. The resulting construct was transformed into DH5 α and plasmid isolated from a large scale preparation.

Since constructs #2C and #2D did not bind CEA (see next section), other chimeric receptor constructs were generated that maintained the transmembrane domain of construct #1 from Fc γ .

-Construction of plasmid #3B

The scFv anti-CEA and Fc γ transmembrane sequence was excised from the plasmid expressing construct #1 (scFv-CEA-Fc γ) and this segment extended by PCR with primers to allow insertion of the sequence into the Bgl II Sal I sites of the retroviral expression plasmid pLNCX-2 (Clontech, Palo Alto, CA). Primers were synthesized to allow the intracellular domain of TNF α RI to be inserted into the Sal I site of the scFv-CEA+TM/pLNCX-2 vector. Constructs were tested for proper orientation within the Sal I site and a properly oriented construct was transformed into DH5 α and plasmid isolated from a large scale preparation.

-Construction of plasmid #3C

The scFv anti-CEA and Fc γ transmembrane sequence was excised from the plasmid expressing construct #1 (scFv-CEA-Fc γ) and this segment extended by PCR with primers to allow insertion of the sequence into the Bgl II Sal I sites of the retroviral expression plasmid pLNCX-2 (Clontech, Palo Alto, CA). Primers were synthesized to allow the intracellular domain of EGFR to be inserted into the Sal I site of the scFv-CEA+TM/pLNCX-2 vector. Constructs were tested for proper orientation within the Sal I site and a properly oriented construct was transformed into DH5 α and plasmid isolated from a large scale preparation.

Plasmids #2C, #2D, #3B, and #3C were sequenced and it was shown that the constructs were in phase. Sequenced constructs were used to generate retroviral vectors.

7. Generation of a retroviral vectors for scFv anti-CEA chimeric receptors

The pLNCX-2 retroviral expression vector containing the scFv-CEA chimeric receptor sequences (#2C, #2D, #3B and #3D) were used to transfect the RetroPack PT67 packaging cell line (Clontech) using LipofectAmine (GibcoBRL, Rockville, MD). Cells resistant to G418 (500ug/ml) were selected for generation of the defective, infectious retrovirus containing the gene for scFv anti-CEA receptors.

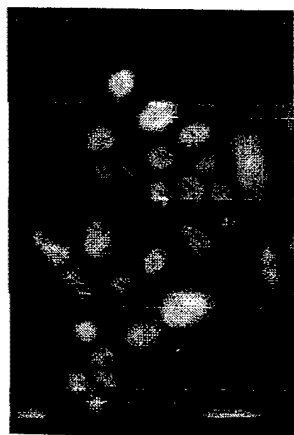
8. Transfection and selection of human keratinocytes and HaCaT cells

Virus from the PT67 cells transfected with the vector containing the scFv anti-CEA chimeric receptor sequences were used to infect HaCaT cells in the presence of polybrene [11]. Stable, transfected HaCaT cells were grown in G418 (600ug/ml) and used for further study.

9. Binding of I-125 CEA to transformed HaCaT cells

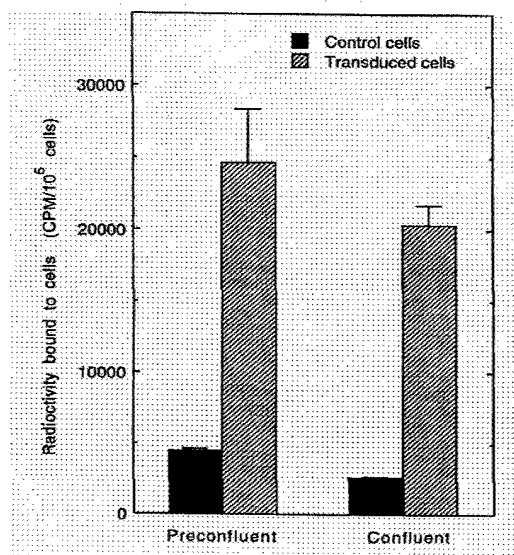
Binding of I-125 CEA was performed as previously carried out for the human keratinocytes expressing construct #1, scFv-CEA Fc γ (Figure 2). Binding of I-125 CEA to scFv-CEA chimeric receptors on HaCaT cells was compared to binding of I-125 CEA to scFc-CEA Fc γ HaCaT cells (positive control with construct #1). No binding of CEA was observed in the scFv-CEA chimeric receptor HaCaT cells but binding was measured to the positive control cells (Figure 3).

Figure 1. Immunofluorescent localization of scFv-CEA—Fc γ to cell surface of keratinocytes.



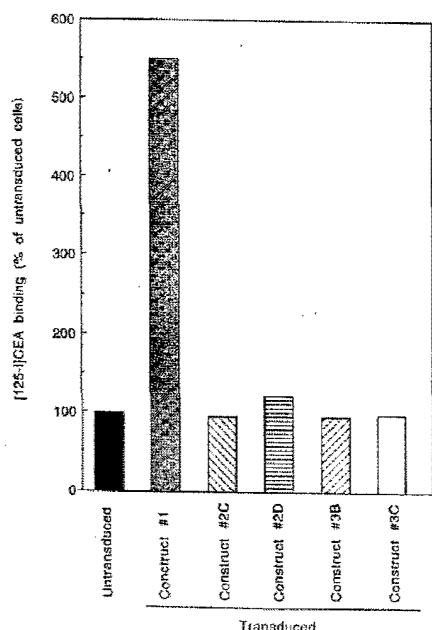
G418-selected keratinocytes transduced with a MMLV retroviral vector expressing the scFv-CEA – Fc γ chimeric receptor were grown on 4-well glass culture slides. After 48hrs. cells were fixed in cold acetone. The presence of the c-myc tag sequence in the extracellular hinge domain of the receptor was detected by staining with anti-c-myc (Sigma) and FITC-anti-mouse IgG (Sigma). Controls, 1 $^{\circ}$ antibody negative and untransduced keratinocytes, had only diffuse background staining (not shown). About 60% of transduced cells displayed bright staining.

Figure 2. Binding of ^{125}I -CEA to Human Keratinocytes Transduced with a Retroviral Vector for Expressing scFv-CEA – Fc- γ .



Keratinocytes were seeded in 4-well dishes and allowed to grow for 48hrs. Cells were seeded at different densities to give preconfluent and confluent cultures. Complete MCDB growth medium was removed and replaced with MCDB containing ^{125}I -CEA for 45 mins at 37°C. After removal of the unbound CEA, cell monolayers were washed twice with PBS and precipitated with 10% TCA. The precipitate was re-dissolved in 0.2M NaOH, 0.2% SDS and radioactivity associated with the cells was determined in a gamma counter. Each bar represents the mean \pm SD for 3 wells divided by the cell number determined by Coulter counter from 2 wells grown under identical conditions.

Figure 3. Binding of ^{125}I -CEA to HaCaT Cells and HaCaT Cells Transduced with a Retroviral Vector for Expressing scFv-CEA –chimeric receptors.



Cells were seeded in 4-well dishes and allowed to grow for 48hrs. Complete MCDB growth medium was removed and replaced with MCDB containing ^{125}I -CEA for 45 mins at 37°C. After removal of the unbound CEA, cell monolayers were washed twice with PBS and precipitated with 10% TCA. The precipitate was re-dissolved in 0.2M NaOH, 0.2% SDS and radioactivity associated with the cells was determined in a gamma counter. Each bar represents the mean \pm SD for 3 wells divided by the cell number determined by Coulter counter from 2 wells grown under identical conditions.

Problems in accomplishing tasks

The fundamental problem we encountered was designing a chimeric receptor that would bind and respond to CEA in vitro. The control construct #1 was expressed on human keratinocytes indicating that expression of a chimeric receptor on cells in culture was no a limitation to this project. However, no other constructs that we generated and introduced into keratinocytes resulted in CEA binding. Even when chimeric receptors were constructed with the CEA - ligand binding domain and the transmembrane domain from the successful construct #1, no CEA binding was observed.

KEY RESEARCH ACCOMPLISHMENTS

In this grant we have learned that:

1. Human keratinocytes in vitro can be engineered to express a chimeric cell surface receptor.
2. Human keratinocytes expressing a chimeric cell surface receptor will recognize and bind a foreign ligand.

REPORTABLE OUTCOMES

Based on the findings generated during the first year of this grant, other applications have been submitted which further explore the development of epidermal biosensors.

Funded application to NCI – Division of Cancer Prevention
STTR Phase I application with Organogenesis Inc.

Publications

NB: Publication and patent applications were submitted prior to award of this grant.

Milstone, L.M. and Schwartz, P.M. Engineering better skin. In: Skin, The Barrier Zone, S. Klaus and J. Hamburger, eds. (in press).

Provisional Patents (Disclosed to Yale University School of Medicine):

Schwartz, P.M. Epidermal Biosensors: Continuous, Early, Sensitive Monitors for Onset of Specific Diseases.

Schwartz, P.M. An Epidermal Biosensor for Carcinoembryonic Antigen.

CONCLUSIONS

The research we have conducted during this grant has allowed us to conclude that human keratinocytes in vitro can be engineered to express a chimeric cell surface receptor and that these modified cells can recognize and bind CEA.

Technical problems arising in the 2nd year have stalled our testing of the scFv-anti-CEA TNF α RI construct. This problem was been identified and solved. A clear plan was outlined to generate alternative chimeric receptors based on the EGF receptor. Five chimeric receptor constructs were introduced into keratinocytes. Only the control construct (#1) displayed any binding to CEA.

We remain confident that we can generate chimeric receptors that can be expressed in keratinocytes that will permit human keratinocytes to recognize and bind tumor antigens. If functioning chimeric receptors can be introduced into human skin cell, then it should be possible to develop effective epidermal biosensors for early, continuous in vivo detection of breast cancer. Such an early detection system is likely to play an important role in the management of breast cancer especially in women who have known risk factors.

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APPENDICES

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in, Skin: The Barrier Zone
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ENGINEERING BETTER SKIN

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Mammalian skin is a remarkably effective barrier. As we have heard over the past several days, parasites and insect vectors have developed a variety of physical tools, enzymes and other molecular strategies needed to breach the skin barrier. Parasites also commonly trick the host and evade detection as they pass through the skin using poorly understood stealth mechanisms. Previous speakers have identified the reasons for these adaptations: the skin is richly endowed with rapid and redundant response mechanisms to resist assault and to repair barrier integrity. In light of its clever design and effective performance, it is with some hesitancy and humility that we have considered ways to improve the epidermis.

Moving genes for the purpose of improving nature, otherwise known as genetic engineering, is changing our views of how we treat disease and where we draw the borders between disease and well being. Current technologies can be used to amplify existing traits or to introduce new traits into virtually any living organism; any tissue can be genetically modified, including skin. Yet there is debate as to whether the outcomes of genetic engineering represent biological improvements. This debate is particularly sharp in higher organisms where we neither can reliably predict all the consequences of our efforts nor can we select and clone our best efforts.

In this review, we provide a brief overview of technical issues relevant to genetic engineering in skin. Our own work reflects the bias that the *engineering* part of genetic engineering implies improvement in the efficiency or activity of an existing function, not the creation of inherently new or distinctive forms or functions. Toward that end, our labs have undertaken efforts to use genetic engineering to augment or broaden the skin's normal function as 1) a biosensor of internal health and well-being, 2) an organ of excretion via desquamation, and 3) the target tissue for restoring genes lost during evolution. We will give examples of how

changes in the skin can alert the clinician to the existence of disease; we will show how augmenting iron loss through desquamation might be used to treat the toxic effects of hemochromatosis; we will indicate how vitamin C might be synthesized in the skin.

Delivering genes to the skin: There are two main approaches to delivering genes to the skin (1-3). The *ex vivo* approach usually involves culturing autologous cells, transducing them with high efficiency viral vectors (e.g. retrovirus, adenovirus, adeno-associated virus), selecting cells for appropriate new gene expression and transplanting selected cells back to the host. The *ex vivo* approach is advantageous when control over the level of expression is important and long-lasting expression is an objective. Its main disadvantage is that it is labor intensive, especially when low efficiency, non-viral methods of transduction are used. The *in vivo* approach utilizes direct transfer of genes into skin via injection, using either a syringe or a biolistic particle accelerator ("gene gun"), or via topical application of naked or liposome-encapsulated DNA. The *in vivo* approach is generally less efficient and predictable than the *ex vivo* approach, but may be advantageous when highly localized and transient gene expression are desirable. For applications related to parasitic diseases, the *in vivo* approach may have distinct advantages.

Why choose keratinocytes?: Gene transfer has been accomplished in skin keratinocytes, fibroblasts, endothelial cells and melanocytes. The advantages of keratinocytes for new gene expression in the skin include: ready accessibility; large potential for population expansion *in vitro*; well-established methods for transplantation; high rate of metabolic activity; high density per mm³; demonstrated ability to secrete peptides into the systemic circulation. For applications related to infectious or parasitic diseases of the skin, keratinocytes would be particularly

attractive targets for genetic manipulation because of their ability to deliver a genetically-encoded toxin or repellant to the outermost limits of the barrier.

Engineering an epidermal biosensor: Skin is a well-recognized, sensitive indicator of internal disease. An engineered, epidermal biosensor would provide an early signal of disease onset, even before traditional signs and symptoms appear. Epidermal biosensors use keratinocytes to detect specific circulating molecules and, by coopting an existing signal transduction pathway, induce a visual or otherwise measurable readout. Epidermal biosensors can be designed to allow continuous monitoring of endogenous molecules (e.g. glucose), or to detect newly appearing molecules associated with a disease (e.g. serum tumor antigen) or an environmental exposure (e.g. toxic chemical). For example, an epidermal biosensor might be designed to detect circulating prostate serum antigen (PSA) in patients at risk for prostate cancer or to detect carcinoembryonic antigen (CEA) in patients at risk for recurrence of bowel cancer. The strategy that we are exploring is to utilize the keratinocytes' ability to initiate an inflammatory response following activation of their cell surface receptors for Fc- γ or Il-1 (4-6). Keratinocytes can be engineered to express chimeric receptors, which utilize the transmembrane/intracellular effector domain of either the native Fc- γ or Il-1 receptors fused to a novel ligand-binding domain. The ligand-binding domain is generated by splicing together the variable domains of heavy and light chains of antibodies known to bind ligands of interest (7). In our work these single chain fragments of the heavy and light chain variable domains (scFv) are chosen to selectively bind circulating tumor antigens such as PSA or CEA. Increasing knowledge about the design and function of chimeric receptors (8,9) and the capacity to develop large libraries of scFv's that bind a wide variety of ligands (7) will be instrumental in developing useful epidermal biosensors. We

are currently testing the hypothesis that CEA interaction with a CEA/Fc- γ chimeric receptor expressed on genetically engineered keratinocytes will initiate intracellular signals that result in a tissue response of erythema or inflammation. Alternatively, it should be possible to design epidermal biosensors for which a readout of oxygen pressure, fluorescence or hyperpigmentation could be measured by a transcutaneous scanning device.

Engineering epidermis for remediation of potentially toxic chemicals: There are two ways in which the epidermis can eliminate potentially toxic materials from the general circulation: by catabolism and return to the circulation of a non-toxic metabolite or by acting as a sink that first traps the toxin intracellularly and then removes it through the normal process of desquamation. Many years ago we calculated that the epidermis had the capacity to degrade large amounts of circulating nucleotides (10). Children with adenosine deaminase deficiency develop combined T and B cell immunodeficiency because of high circulating levels of adenosine. Introduction of the normal adenosine deaminase gene into a small fraction of a patient's deficient keratinocytes should be able to metabolize sufficient circulating adenosine to ameliorate the disease (11). This would represent remediation by epidermal catabolism.

Toxin elimination through desquamation is the second way of engineering keratinocytes for the purpose of dermatoremediation. The epidermis turns over or renews itself every 24-28 days. The keratinocytes that are sloughed from the surface, or desquamated, might be engineered to bind and eliminate or excrete toxins through the process of desquamation. As an example of how this might be accomplished, we have been studying hemochromatosis, a disease of iron overload. Under normal circumstances, absorption of dietary iron is limited by feedback mechanisms in the gut. When too much iron gets into the blood, either through a genetic defect

that leads to unregulated iron absorption or as a result of injections of iron through repeated blood transfusions, iron accumulates and impairs function of critical tissues. Incidentally, the epidermis acts as a biosensor of iron buildup: hyperpigmentation or bronze diabetes is a characteristic, though relatively insensitive cutaneous indicator of hemochromatosis. Under normal circumstances, about 10 percent of absorbed iron is eliminated through desquamation of keratinocytes (12). When more iron is delivered to the skin, as in hemochromatosis, more is eliminated through the skin by desquamation. When skin turns over more rapidly in hyperproliferative diseases such as psoriasis, a greater proportion of absorbed iron is lost through the skin. Clearly the skin has the capacity to accept and eliminate increased amounts of iron. How might we load more iron into the skin?

Iron normally circulates bound to a carrier protein, transferrin. In most tissues, uptake of iron is dependent on a cell surface receptor for transferrin. In epidermis, the transferrin receptor is only expressed on the proliferating basal keratinocytes (13). It is presumed that iron accumulates only in basal keratinocytes; any iron in a suprabasal keratinocyte got there before the cell left the basal layer. We are now testing the hypothesis that overexpression of the transferrin receptor on basal keratinocytes or *de novo* expression of the transferrin receptor on suprabasal keratinocytes can lead to increased accumulation of iron in the epidermis and result in increased iron elimination through desquamation. This would represent dermatoremediation by desquamation.

Restoring genes lost through evolution: During the course of evolution, mutations have inactivated the normal function of several useful genes. For example, the gene for gulonolactone oxidase, the enzyme responsible for vitamin C synthesis (14,15), allows animals other than

primates to make their own vitamin C. Humans and other primates have an inactivating mutation in gulonolactone oxidase and require dietary vitamin C. Correction of the existing inactive gene or introduction of an active gulonolactone oxidase gene into skin could provide a continuous supply of vitamin C to skin, potentially reducing oxidative damage to the skin resulting from carcinogen exposure, UV light or aging (16). Because of the large metabolic capacity of epidermis, restoration of inactive genes in epidermis could have beneficial systemic effects, as well.

In these days of great promise but little practical experience, we can only wonder whether our schemes to engineer better skin will ever match the variety and inventiveness of methods used by parasites to penetrate the skin barrier.

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